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## **Hepatic fat loss in advanced nonalcoholic steatohepatitis: Are alterations in serum adiponectin the cause?**

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## Abstract

Advanced liver fibrosis in nonalcoholic steatohepatitis (NASH) is often accompanied by a reduction in hepatic fat to the point of complete fat loss (burnt-out NASH), but the mechanisms behind this phenomenon have not been elucidated. Adiponectin is raised in cirrhosis of any cause and has potent antisteatotic activity. In this study we examined 65 patients with advanced biopsy-proven NASH (fibrosis stage 3-4) and 54 with mild disease (fibrosis stage 0-1) to determine if disappearance of steatosis correlated with changes in serum adiponectin. All patients had fasting blood tests and anthropometric measures at the time of liver biopsy. Liver fat was accurately quantitated by morphometry. Serum adiponectin was measured by immunoassay. When compared to those with early disease, patients with advanced NASH were more insulin-resistant, viscerally obese, and older, but there was no difference in liver fat content or adiponectin levels. Adiponectin had a significant negative correlation with liver fat percentage in the whole cohort ( $r = -0.28$ ,  $P < 0.01$ ), driven by patients with advanced NASH ( $r = -0.40$ ,  $P < 0.01$ ). In advanced NASH, for each 4  $\mu\text{g/L}$  increase in adiponectin there was an odds ratio OR of 2.0 (95% confidence interval [CI]: 1.3-3.0,  $P < 0.01$ ) for a 5% reduction in hepatic fat. Adiponectin was highly and significantly associated with almost complete hepatic fat loss or burnt-out NASH (12.1 versus 7.4  $\mu\text{g/L}$ ,  $P = 0.001$ ) on multivariate analysis. A relationship between adiponectin, bile acids, and adipocyte fexamine activation was demonstrated in vivo and in vitro, suggestive of hepatocyte-adipocyte crosstalk. Conclusion: Serum adiponectin levels in advanced NASH are independently associated with hepatic fat loss. Adiponectin may in part be responsible for the paradox of burnt-out NASH.

## Introduction

Nonalcoholic steatohepatitis (NASH) is characterized histologically by hepatic steatosis, inflammation, ballooning of hepatocytes, and liver fibrosis.<sup>1</sup> Hepatic steatosis, or fat, is considered intrinsic to the disorder, and a sine qua non of the most commonly used disease classifications.<sup>2-4</sup> It is therefore somewhat of a paradox, and an intriguing but well-replicated observation, that in advanced fibrotic NASH the disease in some patients appears to “burn out” with liver histology revealing little or no discernable fat.<sup>5-7</sup> Indeed, in the 20 years since NASH was first recognized as a common cause of cryptogenic cirrhosis,<sup>7</sup> further studies have shown NASH to be responsible for the majority of such cases,<sup>5, 6</sup> and diagnostic algorithms have been created accordingly.<sup>8, 9</sup> A number of putative mechanisms have been proposed to explain the loss of hepatic fat in advanced NASH including portosystemic shunting,<sup>10</sup> changes in mitochondrial metabolism,<sup>8</sup> vascular changes,<sup>11</sup> and the inflammatory, catabolic state associated with cirrhosis.<sup>12</sup> The exact mechanisms behind this phenomenon, however, have not been elucidated.

Adiponectin, the most abundant human adipocytokine, is intimately associated with hepatic steatosis, acting directly on hepatocytes to upregulate fatty acid oxidation, inhibit fatty acid synthesis, and to improve insulin sensitivity.<sup>13</sup> In obese mice adiponectin treatment is associated with resolution of hepatic steatosis and hepatomegaly,<sup>13</sup> whereas in humans treatment with peroxisome proliferator-activated receptor (PPAR)-gamma ligands such as pioglitazone increase adiponectin levels and reduce liver fat.<sup>14</sup> As expected, adiponectin levels are inversely correlated with steatosis and necroinflammation in NASH, but the relationship with fibrosis is less clear-cut.<sup>15, 16</sup> Adiponectin is increased in cirrhosis of any cause and, importantly, levels incrementally increase between fibrosis stages 3 and 4, as well as Child's stages A and B.<sup>15, 17, 18</sup> Furthermore, adiponectin levels in established cirrhosis are independent of body mass index (BMI) and insulin resistance, but rather, correlate with markers of liver synthetic dysfunction and cholestasis.<sup>17, 18</sup> Given these

observations (fat loss in advanced disease and reports of elevated circulating adiponectin with progressive hepatic fibrosis), we surmised that adiponectin may in part explain hepatic fat loss in late-stage NASH. To test this hypothesis we measured serum adiponectin in 65 NASH patients with advanced fibrosis (fibrosis stage [F]3-4) and a similar number with early disease (F0-1). Because liver histology is semiquantitative, we further examined the relationship between serum adiponectin and accurate quantification of hepatic fat using morphometry of whole liver biopsy cores. We found that adiponectin is independently associated with hepatic fat loss in late stage, compensated NASH and that it is the only independent predictor of burnt-out NASH. Further, we suggest the novel concept that bile acid-mediated hepatocyte-adipocyte crosstalk may mediate the elevations in serum adiponectin in advanced fibrotic liver disease.

## Materials and Methods

### Patients and Data Collection.

We performed a cross-sectional study on 119 adults with biopsy-proven NASH recruited from tertiary liver clinics at Westmead Hospital, Sydney Australia, and the University of Turin Italy. From prospectively collected databases of over 800 consecutive patients, 65 patients with advanced NASH (F3 or 4), had stored serum and liver tissue available for analysis. They were compared to 54 consecutive patients with mild NASH (F0 or 1). Patients with intermediate stage fibrosis (F2) were excluded to ensure a valid comparison between early and advanced disease. Patients were referred for the assessment of abnormal liver tests or hepatic steatosis detected by ultrasonography. In all patients, current and past daily alcohol intake was less than 40 g per week, confirmed by at least two physicians and close family members. All subjects had a normal serum albumin level, prothrombin time, and renal function. To minimize the effects of protein-calorie malnutrition and catabolism from cirrhosis, all patients were Childs Class A. None of the patients were using thiazolidinediones. Secondary causes of steatohepatitis and other causes of liver disease were excluded by appropriate serological and biochemical tests. The study protocol was approved by the Human Ethics Committee of the Western Sydney Area Health Service and the University of Turin and written informed consent was obtained.

### Pathology.

Liver tissues were stained with hematoxylin-eosin, reticulin, and Gomori trichrome stains and scored by an experienced hepatopathologist. The diagnosis of NASH was made according to the method of Brunt et al.<sup>2</sup> Necroinflammatory activity was graded from 0-3 and fibrosis stage from 0-4.<sup>19</sup> A precise liver fat percentage was determined by morphometric analysis of liver core tissue and stained using Gomori trichrome. Slides were examined and photographed using a Leica DMLB microscope with a Spot RT camera (Leica Microsystems, Wetzlar Germany). For each biopsy

images that covered the entire liver core at 40× power were obtained to quantitate fat. Images were then analyzed using ImageJ software (ImageJ, NIH, Bethesda, MD<sup>20</sup>) and the quantity of fat determined as a percentage of the total liver core. Fat quantitated by this method has been shown to correlate highly with liver fat as determined by magnetic resonance spectroscopy and thus is reflective of larger volumes of liver tissue.<sup>21</sup>

#### Clinical and Laboratory Evaluation.

A complete physical examination was performed on each subject on the day of liver biopsy. Anthropometric evaluation included measures of BMI and central obesity (waist and hip circumferences and waist-hip ratio [WHR]). On the morning of liver biopsy, venous blood samples were drawn after an overnight 12-hour fast to determine the levels of serum alanine aminotransferase (ALT), bilirubin, albumin, total cholesterol, triglycerides, glucose, insulin, and adiponectin. Serum insulin was determined by a radioimmunoassay technique (Phadeseph Insulin RIA; Pharmacia and Upjohn Diagnostics, Uppsala, Sweden). Serum adiponectin and leptin levels were measured in duplicate by enzyme-linked immunosorbent assays (Quantikine ELISA; R&D Systems, Minneapolis, MN, and Diagnostic Systems Laboratories, Webster, TX). All additional biochemical tests were performed using conventional automated analyzers within the Department of Clinical Chemistry at Westmead Hospital and the University of Turin. Insulin resistance was calculated by the homeostasis model (HOMA-IR) using the formula:  $\text{HOMA-IR} = \text{fasting insulin (mU/L)} \times \text{plasma glucose (mmol/L)} / 22.5$ .<sup>22</sup>

#### Immunohistochemistry.

Formalin-fixed, paraffin-embedded 4-μ sections were dewaxed, subjected to antigen retrieval using 10 mM sodium citrate buffer, pH 6.0, and treated with DAKO block. Endogenous peroxidase activity was blocked with 0.6% H<sub>2</sub>O<sub>2</sub> for 15 minutes, followed by treatment with an avidin/biotin blocking kit (Vector Laboratories). Sections were then stained overnight with adiponectin (Clone



19F1, Abcam), phospho-activated protein kinase (p-AMPK; Thr172, clone 40H9), and phospho-acetyl-CoA carboxylase (p-ACC; Ser79, antibody #3661) from Cell Signaling and detected with antimouse DAKO Envision or biotin labeled goat antirabbit antibodies (ab6012) and subsequent streptavidin horseradish peroxidase. The site of the antigen was visualized with 3,3'-diaminobenzidine treatment and counterstained with hematoxylin. The specificity of staining was confirmed by positive controls as previously published<sup>23</sup> and negative controls with absent primary antibody.

#### Western Blot and Cell Culture.

3T3-L1 cells were maintained as subconfluent cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Postconfluent 3T3-L1 cells were treated with 220 nM dexamethasone, 100 ng/ml biotin, 1.4 mM insulin, and 500 mM 3-isobutyl-1-methylxanthine for 3 days, followed by incubation with 1.4 mM insulin for 2 days and then maintained for a further 4 days in DMEM containing 10% FCS. Cells were washed once and treated with fexaramine (a generous gift from Prof. Ronald Evans, Salk Institute, La Jolla, CA) or tauroolithocholic acid for 24 hours. Conditioned media were collected, precipitated with trichloroacetic acid, pelleted, washed twice with acetone, air-dried, resuspended in reducing Laemmli buffer, and heated to 95°C for 5 minutes. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting then performed and membranes probed with anti-adiponectin (Affinity BioReagents) and anti-complement C3 (Pierce) antibodies.

#### Serum Bile Acid Measurement.

Individual serum bile acids were quantified by high-performance liquid chromatography-mass spectrometry (LC/MS) using authentic bile acid standards and deuterated internal standards as described.<sup>24</sup> The detection limit for individual bile acids was 10 to 50 nmol/L.

#### Statistical Analysis.

Statistical analysis was carried out using SPSS v. 16.0 (IBM, Armonk, NY). Results are reported as mean  $\pm$  standard deviation (SD) or frequency (percentage) as appropriate. The strength of association between continuous variables was reported using Spearman rank correlations. Student's t tests were used to compare means of continuous variables and  $P < 0.05$  was considered significant throughout. Univariate analysis of variance (ANOVA) was used to examine factors associated with increasing increments of hepatic fat, as this was a categorical variable with multiple endpoints. Multiple ordinal regression analysis was carried out to determine which factors were significant on ANOVA, remained independent predictors for hepatic fat when adjusted for clinically relevant variables such as BMI, WHR, leptin, fibrosis stage, and HOMA-IR. Binary logistic regression with stepwise removal of variables was used to determine the independent associations of almost complete hepatic fat loss. Input variables included those significant on univariate analysis and clinically relevant variables such as BMI, WHR, leptin, bilirubin, platelets, and HOMA-IR.

## Results

The baseline characteristics of the 119 patients studied, 54 with early NASH (F0-1) and 65 with advanced NASH (F3-4) are listed in Table 1. Forty-three percent of patients with advanced NASH had cirrhosis and just under half were males. When compared to those with early NASH, the advanced NASH patients were older, more insulin-resistant, and had higher WHR and prothrombin time ( $P < 0.05$  for all). There was no difference for the two groups in overall BMI, serum leptin, or liver fat percentage. Adiponectin levels were not elevated in those with advanced fibrosis, compared to early disease, even when cirrhotics were considered alone (9.3 versus 8.9  $\mu\text{g/mL}$ ,  $P = 0.7$ ).

### Adiponectin Inversely Correlates with Hepatic Fat in Advanced NASH.

The univariate correlates of hepatic fat and adiponectin are presented in Table 2. There was a significant inverse correlation between serum adiponectin levels and the extent of hepatic fat across the whole NASH cohort ( $r = -0.28$ ,  $P < 0.01$ ), driven by patients with advanced fibrosis ( $r = -0.40$ ,  $P < 0.01$ ). Thus, as hepatic fat declined adiponectin levels significantly increased. Patient age was also associated with hepatic fat, once again primarily in those with advanced, but not mild NASH. In patients with advanced NASH there was no association between liver fat and any of the other key metabolic variables such as BMI, WHR, HOMA-IR, or leptin. In contrast, in those with F0-1 NASH, liver fat and adiponectin were significantly associated with insulin resistance as measured by HOMA-IR ( $r = 0.32$ ,  $r = 0.36$ ,  $P < 0.05$ ). In advanced NASH, serum adiponectin had a significant positive correlation with increasing age, but consistent with previous reports, there was no association with HOMA-IR or markers of adiposity (table 2).

To further elucidate the relationship between hepatic fat and adiponectin in advanced NASH, we subdivided patients based on 5% increments of hepatic fat as follows:  $>15\%$  fat ( $n = 17$ ),  $10.1\%-15\%$  ( $n = 13$ ),  $5.1\%-10\%$  ( $n = 18$ ),  $0\%-5\%$  ( $n = 17$ ), and observed that adiponectin levels

progressively increased as hepatic fat declined. Indeed, for each 4 µg/mL increase in adiponectin there was an odds ratio (OR) of 2.0 (95% confidence interval [CI]: 1.3-3.0, P = 0.002) for a 5% reduction in hepatic fat (Fig. 1, Table 3). BMI, WHR, HOMA-IR, fibrosis stage, and leptin were not predictive of changes in hepatic fat. Adiponectin, along with increasing age, were the only independent predictors of reducing hepatic fat by multiple ordinal regression, even when HOMA-IR, WHR, fibrosis stage, leptin, and BMI were considered (Table 3; OR 1.6, 95% CI: 1.1-2.6, P = 0.03).

#### High Serum Adiponectin Is the Strongest Predictor of Burnt-Out NASH.

We next evaluated the associations with almost complete hepatic fat loss (<5% fat), so called burnt-out NASH, in patients with advanced disease. In this subgroup of 17 patients (26% of cohort) the highest adiponectin was seen, with mean levels of 12.1 as compared to 7.4 µg/mL in the remaining patients (P = 0.001). The only other factor associated with burnt-out NASH was increasing age, whereas interestingly, a nonsignificant trend to higher bilirubin was also noted (Supporting Table 1). When evaluated in a logistic regression model, adiponectin remained an independent predictor of almost complete hepatic fat loss, even when controlled for these factors (Table 4).

#### Serum Adiponectin Correlates with Hepatic Adiponectin Protein Activity.

The described results strongly suggested an association between elevated serum adiponectin and hepatic fat loss; however, causality cannot be inferred. Adiponectin, in part, signals through phosphorylation of AMPK and ACC to reduce lipogenesis. To corroborate our data, we therefore next examined for evidence of a functional consequence of elevated circulating adiponectin by immunostaining for p-AMPK, p-ACC, and adiponectin in liver biopsies from patients with advanced NASH. In patients with high adiponectin and low fat (and consistent with our hypothesis), there was intense adiponectin staining and an increase in granular and cytoplasmic p-AMPK and p-ACC

staining. In contrast, those with low adiponectin and high fat had less intense adiponectin staining and absent or minimal staining for p-AMPK and p-ACC (Fig. 2).

#### Serum Bile Acids Correlate with Serum Adiponectin Levels.

The above observations suggest that increased adiponectin is associated with hepatic fat loss and further that serum adiponectin in late-stage NASH has downstream signaling effects that could mediate liver fat loss. However, as circulating adiponectin is produced by adipose tissue, we therefore hypothesized that in late-stage NASH the liver must signal to the adipocyte to mediate adiponectin synthesis. Bile acids are the most sensitive marker of liver injury, are increased with progressive liver fibrosis,<sup>25</sup> and are also known to modulate adipocyte behavior.<sup>26</sup> In Table 4 we have shown a nonsignificant trend to higher bilirubin, which closely parallels elevations in serum bile acids. We therefore measured the serum concentrations of individual bile acids in a subset of 28 patients with advanced NASH by LC/MS. By linear regression, levels of deoxycholic acid (DCA) and glycine conjugated DCA paralleled the increase in adiponectin (DCA:  $r = 0.51$ ,  $P < 0.01$ , G-DCA:  $r = 0.49$ ,  $P < 0.01$ ), but not cholic acid (CA), chenodeoxycholic acid (CDCA), or ursodeoxycholic acid (UDCA).

In a final experiment, to test the role of bile acids on adiponectin expression we treated differentiated 3T3-L1 adipocytes with fexaramine (FXR agonist) or tauroolithocholic acid (TGR-5 agonist) and examined the cell culture supernatant for adiponectin protein. We found that both fexaramine and tauroolithocholic acid increased adiponectin protein secretion greater than 10-fold (Fig. 3). These data suggest that bile acids act directly to regulate adiponectin synthesis in adipocytes.

## Discussion

One of the most intriguing and unanswered questions in clinical hepatology concerns the observation that liver fat loss often accompanies advanced fibrosis and cirrhosis, something that delayed the linkage of NASH to cryptogenic cirrhosis for many years. In this study we show for the first time that alterations in serum adiponectin may provide an explanation for this phenomenon and suggest a novel mechanism by which this might occur (Fig. 4). In well-characterized patients with biopsy proven NASH, we show that (1) circulating adiponectin levels have an inverse correlation with hepatic fat content in those with advanced disease; (2) as hepatic fat declines with advanced fibrosis, adiponectin levels progressively rise, independent of its usual metabolic associations, viz. insulin resistance, leptin, BMI, and WHR; (3) elevated serum adiponectin is significantly and independently associated with almost complete hepatic fat loss, so-called “burnt-out” NASH, even when controlled for patient age and markers of liver synthetic dysfunction; (4) circulating adiponectin in advanced NASH is associated with activation of its downstream signaling in the liver; (5) bile acids in late-stage NASH that are ligands for the bile acid receptors FXR and TGR527 are elevated in patients; and finally (6) that this elevation in circulating bile acids may be responsible for the secretion of adiponectin by adipocytes in advanced liver disease (Fig 4).

Adiponectin is a key player in the pathogenesis of NASH-related steatosis, with an intimate association between hypoadiponectinemia, increasing steatosis grade, and the transformation from simple steatosis to NASH.<sup>16, 28</sup> Acting predominantly by way of the hepatic adiponectin receptor 2 (AdipoR2), elevated levels of adiponectin are profoundly antisteatotic, an effect mediated by stimulation of PPAR- $\alpha$  with an increase in fatty acid  $\beta$ -oxidation, and inhibition of fatty acid synthesis by way of SREBP-1c.<sup>13</sup> It is now well established that adiponectin is elevated in advanced liver disease and cirrhosis of any cause, although most evidence exists for viral, autoimmune, cholestatic, and alcohol-related disease.<sup>15, 17, 18</sup> A single report has directly linked

NASH cirrhosis with elevated adiponectin,<sup>29</sup> but others have included numerous patients with cryptogenic cirrhosis, in whom NASH is highly probable.<sup>5, 6</sup>

In the present study, adiponectin levels were not significantly elevated in those with advanced-stage NASH fibrosis/cirrhosis when compared to those with early disease. One might have expected the levels to be lower in patients with advanced NASH who were more insulin resistant and obese than those with early disease, but it is established that adiponectin levels in cirrhosis do not correlate with insulin resistance, dyslipidemia, or obesity.<sup>17, 18</sup> The unaltered levels of adiponectin in late compared to early disease is in part a deliberate consequence of our strict selection criteria, wherein we excluded (1) all patients with markers of liver synthetic dysfunction such as abnormal prothrombin time, albumin, or bilirubin, and (2) those with Child's B and C cirrhosis. Thus, we were able to exclude elevations due to these confounders known to be associated with increased adiponectin, and further strengthen our hypothesis.<sup>17, 18</sup> Adiponectin levels are also lower in patients with nonalcoholic fatty liver disease (NAFLD) compared to other liver diseases<sup>29</sup> and levels decline further with increasing necroinflammation and fibrosis. Thus, the finding of similar adiponectin levels for our two groups is in keeping with a relative elevation of adiponectin, similar to that seen in other forms of cirrhosis. Taken together, these findings suggest that physiological regulation of adiponectin is dramatically altered in patients with advanced-stage liver disease compared to the situation in healthy volunteers, diabetes, or early liver disease.<sup>16, 30</sup>

A number of mechanisms have been hypothesized to explain the relative elevation in adiponectin with progressive fibrosis, including an imbalance between adiponectin production and hepatic extraction,<sup>18, 31</sup> a protective antiinflammatory mechanism in the chronic inflammatory state of cirrhosis,<sup>18</sup> and an increase in true hepatocyte or hepatic stellate cell adiponectin production.<sup>17, 32</sup> Because the highest levels of adiponectin are seen in patients with advanced cholestatic liver disease, reduced biliary excretion of adiponectin may also be important.<sup>15, 17, 33</sup> This theory is

supported by bile duct ligation studies in mice where dramatic increases in serum adiponectin were seen over time, and the detection of adiponectin in the bile of human subjects with severe cholestasis.<sup>15</sup> None of the patients included in this study were severely catabolic or clinically had cholestasis (elevations in bilirubin), which raised the intriguing question as to why adiponectin would be elevated in our cohort and whether there could be a link between hepatocyte dysfunction and adiponectin production by adipose tissues.

Bile acids are sensitive markers of liver dysfunction, play an important part in metabolic signaling,<sup>34, 35</sup> and are well documented to be increased with advancing liver fibrosis.<sup>25</sup> Hence, our observation that elevation in specific circulating bile acids may be responsible for the secretion of adiponectin by adipocytes was not altogether surprising. If our hypothesis is correct, then hepatocyte-adipocyte crosstalk mediated by bile acids and their receptors could explain our observations and drive adipocyte-mediated adiponectin production in advanced liver disease. Bile acid receptors such as FXR and TGR-5 are expressed in adipose tissue and their activation has been associated with improved insulin sensitivity and metabolic homeostasis.<sup>26, 36</sup> Thus, our final in vitro data demonstrating robust up-regulation of adiponectin secretion in adipocytes treated with bile acid receptor agonists provides direct confirmation of this hypothesis.

A number of alternate mechanisms have previously been proposed to explain hepatic fat loss in advanced NASH, but none have been examined in any detail in a NASH cohort. Cirrhosis is a chronic inflammatory, catabolic state characterized by increased resting energy expenditure, cachexia, and elevated plasma levels of proinflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor alpha (TNF- $\alpha$ ), and IL-1.<sup>12, 18</sup> Additionally, there is increased utilization of systemic fat stores for energy generation,<sup>12</sup> and hepatic fat may be similarly affected. All of these changes tend to be most marked in the end stages of cirrhosis and would not explain the fat loss that is seen in well-compensated patients such as those in our study, or those in previous reports.<sup>7</sup>



Furthermore, in reports where paired biopsy samples have been studied patients with hepatic fat loss tended to have gained, rather than lost weight.<sup>7, 37, 38</sup> Circulatory changes that occur in advanced liver disease have also been implicated in hepatic fat loss. It is known, for example, that focal fatty sparing in steatotic livers can result from aberrant portal venous drainage, which in turn reduces hepatocyte exposure to insulin and triglycerides.<sup>39</sup> Similarly, shunting between the portal and systemic circulations in advanced liver disease has been shown to alter hepatocyte exposure not only to insulin and triglycerides, but also to free fatty acids, lipoproteins, and precursors of gluconeogenesis.<sup>10</sup> Changes at a cellular level such as a loss of sinusoidal fenestrations,<sup>11</sup> altered mitochondrial function,<sup>8</sup> or liver repopulation with oval cells<sup>40</sup> have also been suggested to reduce hepatic fat, although the data to support any of these theories remain scant, and we believe less plausible in the present cohort. Intriguingly, there is increasing evidence to suggest hepatic fat loss also occurs in advanced hepatitis C virus (HCV)<sup>41</sup> and the phenomena is likely to be replicated in the late stages of all steatotic liver disease. Adiponectin is inversely associated with steatosis in genotype 4 HCV,<sup>42</sup> but is elevated in all genotypes once cirrhosis is established,<sup>15, 18</sup> Hence, one could speculate that adiponectin may also be responsible for hepatic fat loss that is seen with advanced HCV.

One limitation of this study relates to the cross-sectional nature of the data and therefore the difficulty in inferring cause and effect. Ideally one would like to show longitudinal changes in both liver fat and adiponectin levels over many timepoints to be sure the two were directly related. The invasive nature of liver biopsy, logistic and ethical constraints, and the numerous additional confounding interactions that occur over time mean this approach is not feasible. Hence, we analyzed our data in a number of ways to strengthen and validate the association between adiponectin and hepatic fat loss. We showed the association to hold true for quintiles of hepatic fat and for those with almost total fat loss and, importantly, that it was independent of key

metabolic variables, patient age, and liver dysfunction. We also confirmed that serum adiponectin was correlated with hepatic adiponectin protein activity based on liver blots for downstream enzymes. Finally, in animal studies of NASH and in carbon tetrachloride-induced liver fibrosis exogenous adiponectin administration reduces hepatic steatosis, an expected consequence of the known physiological actions of this protein.<sup>13</sup>

In conclusion, circulating adiponectin levels in compensated late-stage NASH are significantly associated with hepatic fat loss, independent of metabolic or liver dysfunction. Our data in toto support the notion of bile acid-mediated hepatocyte-adipocyte crosstalk, leading to elevations of circulating adiponectin, which in turn may in part be responsible for the paradox of burnt-out NASH.

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## Figure Legends

**Figure 1.** Serum adiponectin levels compared to 5% increments in hepatic fat. Low serum adiponectin levels are seen in patients with high hepatic fat. As the hepatic fat percent declines there is a significant increase in adiponectin levels.

**Figure 2.** Immunohistochemistry: Liver sections were stained with p-ACC, p-AMPK, and adiponectin to demonstrate adiponectin activity. (B,C) Absent or minimal staining for p-AMPK and p-ACC in patients with low serum adiponectin and high fat. (E,F) Increased cytoplasmic granular staining with pericanilicular association for p-AMPK and p-ACC in patients with high serum adiponectin and low fat. (H,J) Increased intensity of adiponectin staining in patients with high adiponectin and low fat (J) compared to those with low adiponectin and high fat (H). (A,D,G,I) Negative controls with absent primary antibody (all images 400×).

**Figure 3.** FXR and TGR-5 receptor agonists induce adiponectin secretion by adipocytes *in vitro*. Reducing SDS electrophoresis of total adiponectin protein after (A), fexaramine (Fex) and (B), tauroolithocholic acid (T-LCA) at the indicated concentrations ( $\mu$ M). (C) Loading control (Complement C3).

**Figure 4.** Adiponectin is elevated in advanced liver disease and is associated with steatosis resolution in NASH. In health, adiponectin is regulated by metabolic factors such as IR, adiposity, and dyslipidemia, but these are not important in advanced liver disease. We propose that hepatocyte-adipocyte crosstalk by way of bile acids may directly stimulate adiponectin production.



**Table 1. Baseline Characteristics of the Patients with NASH**

Baseline Characteristics	NASH F0-1 (n=54)	NASH F3-4 (n=65)	P-value
Cirrhosis	—	28 (43%)	—
Male gender	31 (57%)	32 (49%)	0.3
Age (years)	47.9 (11.6)	53 (12.8)	0.03
BMI (kg/m <sup>2</sup> )	30.3 (4.6)	31.9 (5.6)	0.09
Waist-hip ratio	0.97 (0.08)	1.01 (0.07)	0.02
HOMA-IR	4.9 (3.1)	6.9 (4.0)	<0.01
ALT (IU/mL)	78.4 (44.3)	77 (55)	0.9
Bilirubin (mmol/L)	14.1 (7.8)	14.7 (6.7)	0.6
Platelets (x10 <sup>9</sup> /mL)	266 (66)	199 (80)	<0.01
Prothrombin Time (seconds)	12 (1.0)	14 (3.2)	<0.01
Adiponectin (µg/mL)	8.9 (6.0)	8.6 (5.2)	0.8
Leptin (ng/mL)	30.7 (26.1)	40.1 (33.3)	0.1
Liver fat %	9.7 (6.7)	10.4 (7.1)	0.6

Variables are reported as frequency (percentage) or mean (SD) as appropriate.

BMI; body mass index, WHR; waist hip ratio, HOMA-IR; homeostasis model assessment of insulin resistance, ALT; alanine transaminase.

**Table 2. Univariate Correlations of Hepatic Fat and Serum Adiponectin Levels in Mild and Advanced NASH**

	Adiponectin	Leptin	WHR	BMI	Age	HOMA-IR	ALT	Bilirubin	Platelets
NASH Cohort (n=119)									
Hepatic fat %	-0.28**	0.05	0.04	0.20	-0.33**	0.15	0.14	-0.03	0.10
Adiponectin µg/mL	—	0.02	0.10	-0.11	0.17	-0.23*	-0.06	0.10	0.01
Mild NASH (F0-1, n=54)									
Hepatic fat %	-0.13	0.06	0.04	0.10	-0.19	0.32*	0.05	-0.05	0.09
Adiponectin µg/mL	—	-0.10	-0.10	-0.21	-0.03	-0.36**	-0.05	0.19	0.17
Advanced NASH (F3-4, n=65)									
Hepatic fat %	-0.40**	0.12	0.04	-0.04	-0.45**	0.03	0.21	-0.04	0.15
Adiponectin µg/mL	—	0.12	0.16	0.01	0.33**	-0.10	-0.10	0.04	

\*  $P < 0.05$ ,

\*\*  $P < 0.01$ , WHR; waist hip ratio, BMI; body mass index, HOMA-IR; homeostasis model assessment of insulin resistance, ALT; alanine transaminase.

**Table 3. Multivariate Correlations of Hepatic Fat in Advanced NASH**

	Unadjusted		Best Fitting Model*	
	Odds Ratio (95% CI)	P- value	Odds Ratio (95% CI)	P- value
Hepatic fat grade (5% increments)				
Adiponectin (per 4 µg/L increase)	2.0 (1.3–3.0)	0.002	1.6 (1.1–2.6)	0.03
Age (per 5-year increase)	1.5 (1.2–1.8)	0.001	1.4 (1.1–1.7)	0.002

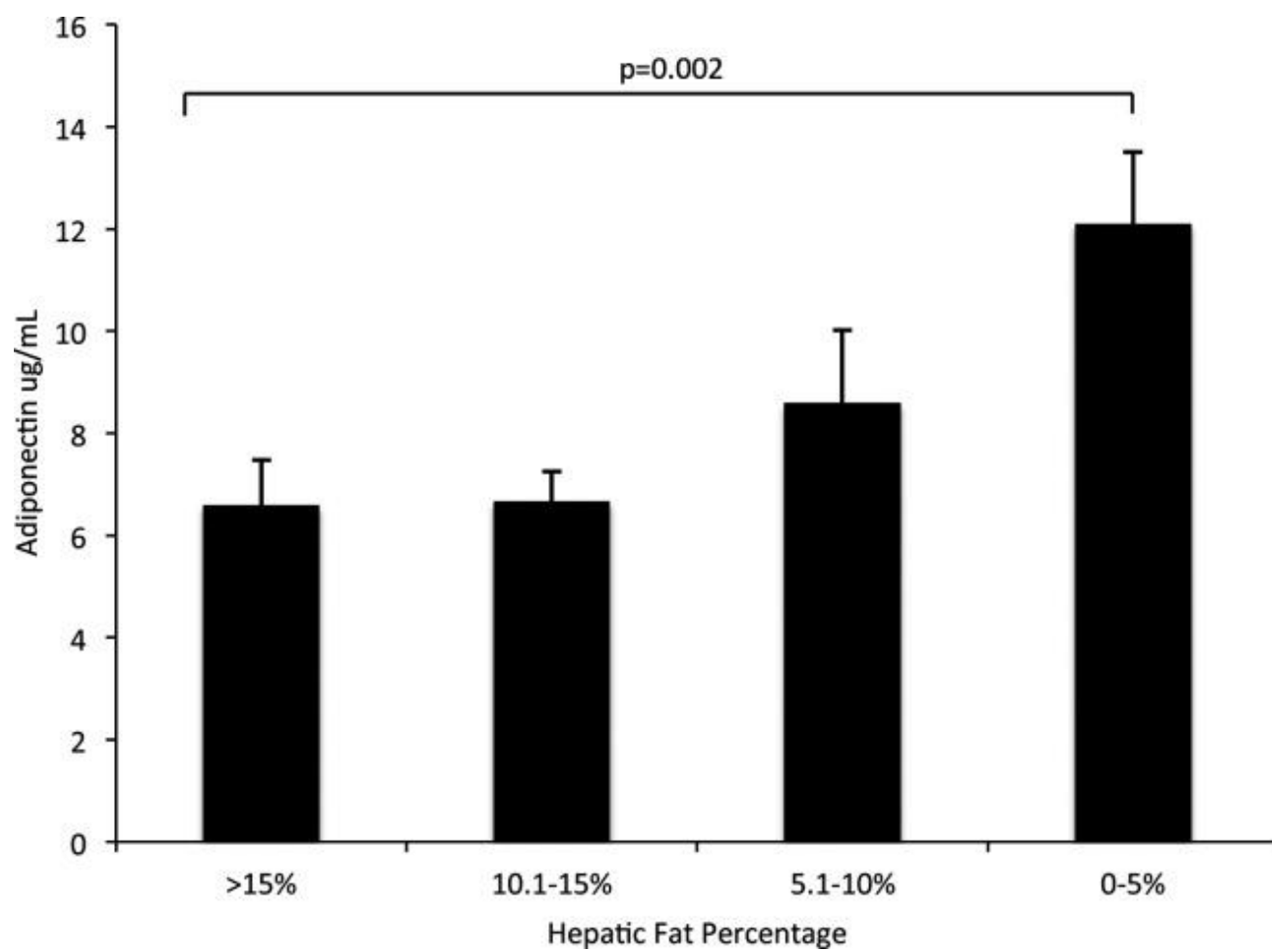


Figure 1

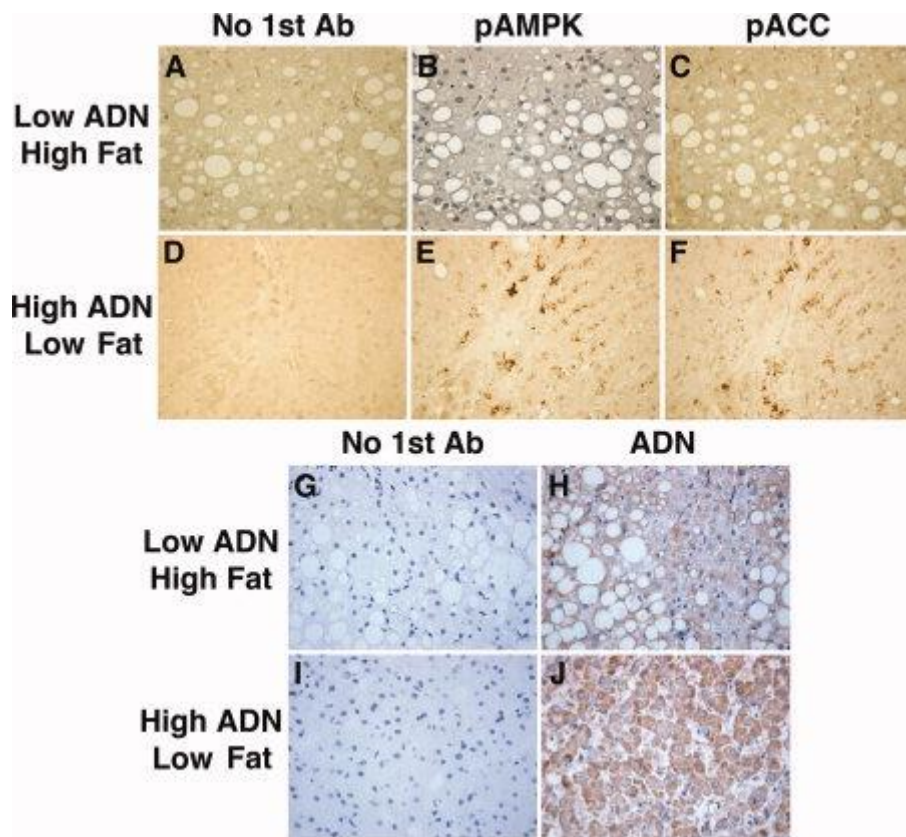


Figure 2

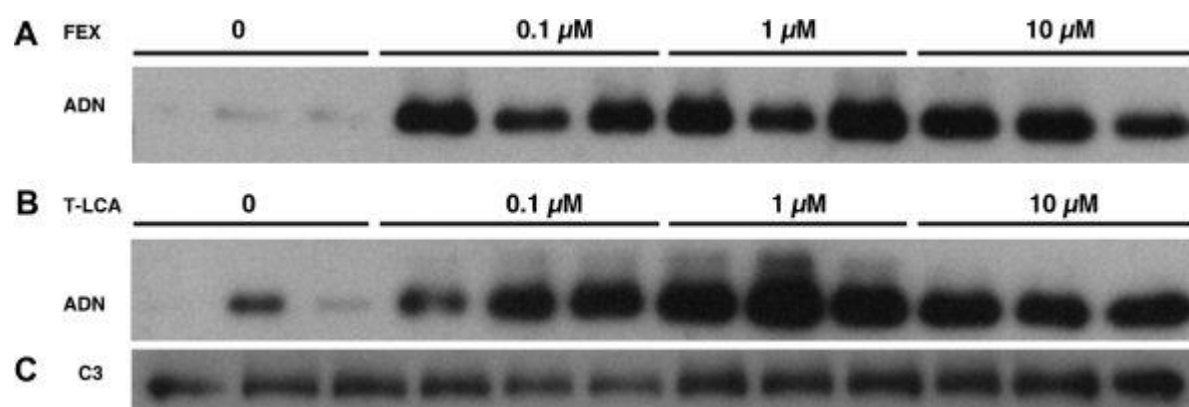


Figure 3

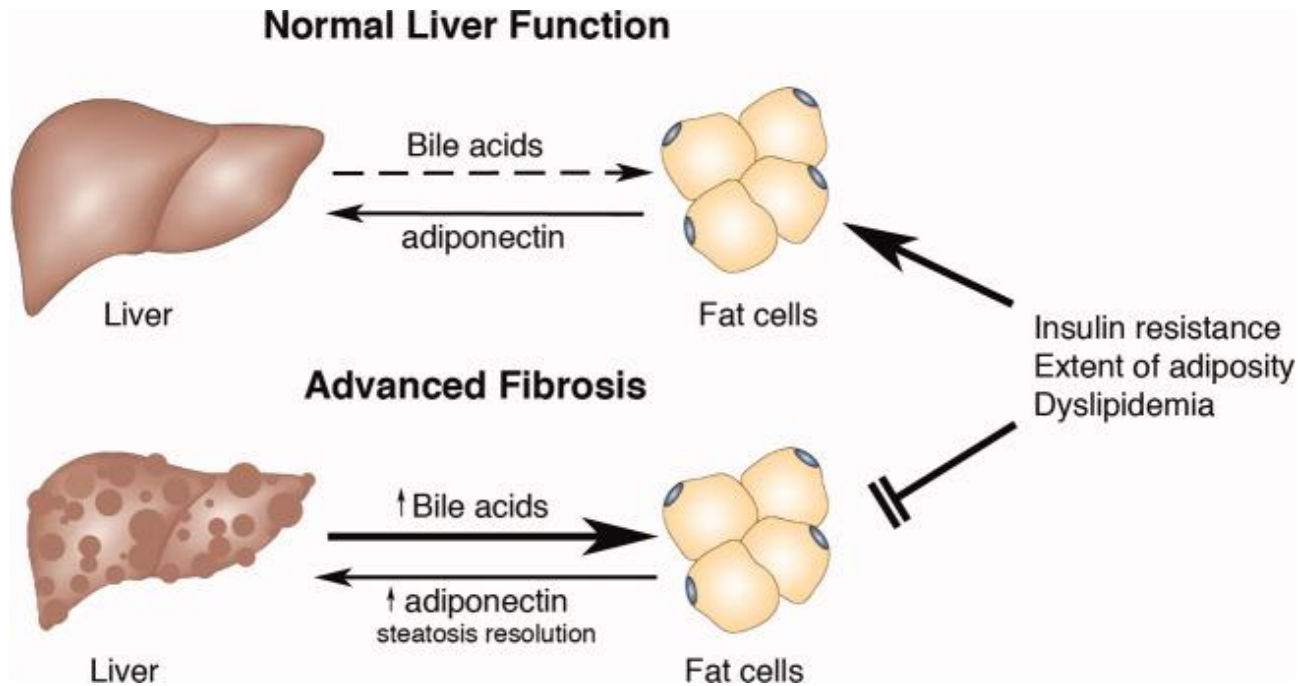


Figure 4

**Supporting Table 1. Univariate correlates for burnt-out NASH (<5% fat)**

Factor	Hepatic fat < 5% (n=17)	Hepatic fat ≥ 5% (n=48)	P-value
Adiponectin (ug/mL)	12.1 (5.8)	7.4 (4.4)	0.001
Age (years)	58.1 (11.3)	50.1 (12.9)	0.05
Bilirubin (mmol/L)	17.6 (9.4)	13.7 (5.2)	0.08
Male gender	8 (47%)	24 (50%)	0.84
BMI (kg/m <sup>2</sup> )	32.3 (6.8)	31.8 (5.2)	0.79
Waist-Hip Ratio	0.99 (0.07)	1.01 (0.07)	0.29
HOMA-IR	6.2 (3.9)	7.0 (4.2)	0.48
ALT (IU/mL)	80 (87)	76 (39)	0.76
Platelets (x10 <sup>9</sup> /mL)	176 (46)	207 (88)	0.38
Prothrombin Time (seconds)	13.4 (4.4)	13.7 (2.8)	0.76
Leptin (ng/mL)	39.8 (37.4)	40.6 (32.2)	0.94

Values are expressed as mean (SD) or frequency (%) as appropriate